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(54) Hybridization assay employing labeled pairs of hybrid binding reagents.

(57) A nucleic acid hybridization assay wherein the hybrid formed with the probe has binding sites for two specific binding reagents, one of which comprises a first label and the other a second label, interaction of the first and second labels providing a detectable response which is measurably different when the two labeled reagents are both bound to the same hybrid compared to when the two labeled reagents are not so bound. The formation of the hybrid assay product brings the two labels within a proximate interaction distance of one another, e.g., as in the cases of sequential catalyst (enzyme) interaction and energy transfer. Since the labels provide a response which is distinguishable when they are associated with a hybridized probe, no separation step is required.

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HYBRIDIZATION ASSAY EMPLOYING
LABELED PAIRS OF HYBRID BINDING REAGENTS

FIELD OF THE INVENTION

This invention relates to nucleic acid
hybridization assay methods and reagent systems for
detecting specific polynucleotide sequences. The
principle of nucleic acid hybridization assays was
developed by workers in the recombinant DNA field
as a means for determining and isolating particular
polynucleotide base sequences of interest. It was
found that single stranded nucleic acids, e.g., DNA
and RNA, such as obtained by denaturing their
double stranded forms, will hybridize or recombine
under appropriate conditions with complementary
single stranded nucleic acids. By labeling such
complementary probe nucleic acids with some readily
detectable chemical group, it was then made
possible to detect the presence of any
polynucleotide sequence of interest in a test
medium containing sample nucleic acids in single
stranded form.

In addition to the recombinant DNA field, the
analytical hybridization technique can be applied
to the detection of polynucleotides of importance
in the fields of human and veterinary medicine,
agriculture, and food science, among others. In
particular, the technique can be used to detect and

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identify etiological agents such as bacteria and viruses, to screen bacteria for antibiotic resistance, to aid in the diagnosis of genetic disorders such as sickle cell anemia and thalassemia, and to detect cancerous cells. A general review of the technique and its present and future significance is provided in Biotechnology (August 1983), pp. 471-478.

INFORMATION DISCLOSURE

The following information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the following information constitutes prior art against the present invention.

The state-of-the-art nucleic acid hybridization assay techniques generally involve a separation of hybridized and unhybridized labeled probe. This required separation step is usually facilitated by immobilizing either the sample nucleic acids or the nucleic acid probe on a solid support. Commonly, hybridization between particular base sequences or genes of interest in the sample nucleic acids and a labeled form of the probe nucleic acid is detected by separating the solid support from the remaining reaction mixture which contains unhybridized probe, followed by detection of the label on the solid support.

There are continuing efforts to simplify the analytical procedure for performing nucleic acid

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hybridization assays. A primary goal of these
fforts is to reduce the complexity of the
procedure to the point that it can be conveniently
and routinely performed in clinical laboratories.

5   The necessity of a separation step seriously
impedes the progress of these efforts. The
separation step requires considerable expertise in
order to be accomplished in an analytically
reproducible manner and is a physical manipulation

10 not readily automated or suited to high volume
testing.

Moreover, in conventional methods involving
the immobilization of sample nucleic acids, two
significant difficulties are encountered. Firstly,

15 the procedures required to accomplish
immobilization are generally time consuming and add
a further step which is undesirable for routine use
of the technique in a clinical laboratory.
Secondly, proteins and other materials in the

20 heterogeneous sample, particularly in the case of
clinical samples, can interfere with the
immobilization process.

As alternatives to immobilizing sample nucleic
acids and adding labeled probe, one can use an

25 immobilized probe and label the sample nucleic
acids *in situ*, or one can use a dual hybridization
technique requiring two probes, one of which is
immobilized and the other labeled. The former
alternative, however, is even less desirable since

30 the *in situ* labeling of the sample nucleic acids
requires a high degree of technical skill which is
not routinely found in clinical technicians and
there are no simple, reliable methods for
monitoring the labeling yield, which can be a

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significant problem if the labeling media contain
variable amounts of inhibitors of the labeling
reaction. The dual hybridization technique has the
disadvantages of requiring an additional reagent
5   and incubation step and the kinetics of the
hybridization reaction can be slow and inefficient.
The accuracy of the assay can also be variable if
the complementarity of the two probes with the
sample sequence is variable.

10   Some of the problems discussed above are
solved by employing an immobilized RNA probe and
detecting resulting immobilized DNA·RNA or RNA·RNA
hybrids with a labeled specific anti-hybrid
antibody [see commonly assigned U.S. Patent
15   Application Serial No. 616,132, filed June 1,
1984]. This technique still requires a separation
step and thus has the disadvantages common to all
hybridization techniques that require a separation
step as discussed above.

20   U.S. Patent Nos. 3,996,345; 4,233,402; and
4,275,149 describe assays to detect antigens by
immunoassay techniques involving enzyme pair and
energy transfer interactions.

        European Patent Application No. 70,685
25   proposes a hybridization assay technique that
dispenses with the need to physically separate
hybridized from unhybridized probe. It is proposed
to employ a pair of probes which hybridize to
contiguous regions on a polynucleotide sequence of
30   interest and to label one probe with a
chemiluminescent catalyst such as the enzyme
peroxidase and the other with an absorber molecule
for the chemiluminescent emission. The catalyst
and absorber labels must be situated near the

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contiguous terminal ends of the respective probes
such that upon hybridization th re is observed
quenching of the chemiluminescent emission by
energy tranfer to the absorber molecule. In order
to perform such an assay, one must be able to
controllably synthesize two critical probe reagents
such that the respective labels are brought into a
quenching orientation upon hybridization to the
sample nucleic acid and without affecting the
affinity of the respectively labeled probe segments
to actually undergo hybridization.

SUMMARY OF THE INVENTION

A nucleic acid hybridization assay has now
been devised based on the use of proximal
interacting labeled pairs which yield a detectable
signal that is measurably different for the
hybridized probe compared to unhybridized probe.
In this way there is no need to separate hybridized
and unhybridized probe, greatly facilitating the
performance and automation of the assay. In
addition, the assay signal is nonradioisotopic in
nature thereby meeting another criterion of assay
convenience, the use of detection systems not
involving radioactivity.

According to the present invention, specific
polynucleotide sequences are detected in a test
sample by forming a hybrid between the sequence to
be detected and a nucleic acid probe having a
complementary sequence such that the resulting
hybrid possesses binding sites for two different
specific binding reagents. One of such binding
reagents comprises a first label and the other a

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second label where interaction between the two
labels provides a detectable r sponse which is
measurably different, either in a positive or a
negative sense, when the two labeled reagents are
5   bound to the same hybrid compared to when not so
bound. When bound to the same hybrid the two
labels are brought to within a proximate
interaction distance of one another, thereby
substantially increasing signal affecting
10   interactions between the two labels compared to the
relatively less frequent interactions occurring in
the bulk solution between the free diffusible
labeled reagents.

A preferred interaction between the two labels
15   is a sequential interaction wherein the first label
participates in a first chemical reaction to
produce a diffusible product that is a participant
in a second chemical reaction with the second label
to produce a detectable product. It is especially
20   preferred that the first and second labels be
catalysts, e.g., enzymes, for the first and second
chemical reactions, respectively. For example,
where the first label is glucose oxidase and the
second is peroxidase, hydrogen peroxide produced by
25   action of glucose oxidase on glucose diffuses to
the peroxidase label to be detectable as an optical
signal in the presence of suitable indicator dye
compositions. Another preferred labeling pair is
that involving energy transfer interaction such as
30   between a fluorescer or luminescer and a quencher
for the photoemission of the first label.

With regard to the nature of the specific
binding agents involved, in principle at least one
of the two must bind to a site which is unique to

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the hybrid formed in the assay compared to
unhybridized nucleic acids since signal generation
must be dependent upon the formation of such
hybrid. Preferably then, such specific binding
5   reagent will be an antibody selective for the
hybrid over the single stranded nucleic acids
present in the assay mixture. A variety of second
binding agents can be used as is detailed below.

The present invention is characterized by a
10   number of significant advantages. In addition to
the principal advantage of elimination of the
separation step, there is no requirement to
immobilize either sample or probe nucleic acids
which gives rise to nonspecific binding and
15   reproducibility problems in commonly used
hybridization techniques. Further, the
hybridization kinetics are substantially faster in
solution compared to systems with one strand of the
hybridizable pair immobilized. An additional
20   advantage is that the assay can be performed
without wash steps. The assay reagents can be
sequentially added to the hybridization medium
without the need to wash insoluble support
materials.

25   Another significant feature of the present
invention is that the detection systems involved
can be particularly efficient since the double
stranded duplexes can contain many binding sites
for the first and second labeled reagents. This
30   results in large amounts of the first and second
labels becoming' assembled into their interactive
configuration per unit of hybridized probe. In
considering antibodies to RNA·DNA or RNA·RNA
hybrids, one labeled antibody can bind for

approximately each 10 bas pairs of the hybrid. If
the probe is for example 500 bases long, 40-50
antibodies could bind. Ligands recognized by
labeled binding proteins can be introduced into
5   probes at levels of one ligand per 5-20 bases. The
presence of multiple binding sites on hybrids can
be used advantageously when low levels of hybrid
must be detected.

BRIEF DESCRIPTION OF THE DRAWINGS

10   Figs. 1-3 are schematic illustrations of
preferred methods for performing the present
invention. These methods are described in detail
below.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

15   The use of nucleic acid hybridization as an
analytical tool is based fundamentally on the
double-stranded, duplex structure of DNA. The
hydrogen bonds between the purine and pyrimidine
bases of the respective strands in double-stranded
20   DNA can be reversibly broken. The two
complementary single strands of DNA resulting from
this "melting" or "denaturation" of DNA will
associate (sometimes referred to as reannealing or
hybridization) to reform the duplexed structure.
25   As is now well known in the art, contact of a first
single stranded nucleic acid, either DNA or RNA,
which comprises a base sequence sufficiently
complementary to (i.e., "homologous with") a second
single stranded nucleic acid under appropriate
30   conditions, will result in the formation of

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DNA·DNA, RNA·DNA, or RNA·RNA hybrids, as the case
may be.

The Probe

The probe will comprise at least one single
stranded base sequence substantially complementary
to or homologous with the sequence to be detected.
However, such base sequence need not be a single
continuous polynucleotide segment, but can be
comprised of two or more individual segments
interrupted by nonhomologous sequences. These
nonhomologous sequences can be linear, or they can
be self-complementary and form hairpin loops. In
addition, the homologous region of the probe can be
flanked at the 3'- and 5'-termini by nonhomologous
sequences, such as those comprising the DNA or RNA
of a vector into which the homologous sequence had
been inserted for propagation. In either instance,
the probe as presented as an analytical reagent
will exhibit detectable hybridization at one or
more points with sample nucleic acids of interest.
Linear or circular single stranded polynucleotides
can be used as the probe element, with major or
minor portions being duplexed with a complementary
polynucleotide strand or strands, provided that the
critical homologous segment or segments are in
single stranded form and available for
hybridization with sample DNA or RNA. It will
generally be preferred to employ probes which are
substantially in single stranded form. The
preparation of a suitable probe for a particular
assay is a matter of routine skill in the art.

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Labeling Pairs

According to the principle of the present
invention, hybridization dependent upon the
presence of the polynucleotide sequence of interest
5   and binding of the labeled reagents to the hybrid
results in the bringing together of the two labels
within a certain distance such that the signal
produced by their interaction is measurably
different from that produced by their encounters
10   during simple diffusion in the bulk assay medium.
Various interaction phenomena can be applied to the
present invention. The interaction can be
chemical, physical, or electrical, or combinations
of these forces. The environment of the labels
15   which is created upon hybridization and binding of
the labeled reagents to the formed hybrid, herein
referred to as the bound hybrid environment, must
be distinctively different in at least one critical
aspect from the bulk medium. Since hybridization
20   determines the proportion of labels which result in
the localized hybrid environment compared to the
bulk phase, the resulting signal response is
dependent upon the presence of the sequence to be
determined in the assay medium.
25   A preferred interaction between the two labels
involves two chemical reactions wherein one label
participates in the first reaction to produce a
diffusible mediator product which participates in
the second reaction with the second label to yield
30   a detectable product. The microenvironment of the
bound hybrid will thus contain a higher localized
concentration of the mediator product, so as to
increase the rate of the signal producing second

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label reaction, than the bulk solution. The two
labels, r spectiv ly, can participate in the
reactions as reactants or, as is particularly
preferred, catalysts. Any involved catalysis can
5   be either enzymatic or nonenzymatic.

A variety of enzymes and catalysts can be
applied to the present invention and their
selection will be a matter of choice to one working
in the field. Useful enzymes for the first
10   reaction include the oxidoreductases, particularly
those involving nucleotides such as nicotinamide
adenine dinucleotide (NAD) or its reduced form
(NADH), or adenosine triphosphate (ATP) as
cofactors or those producing hydrogen peroxide or
15   other small diffusible products. A few examples
are alcohol dehydrogenase, glycerol dehydrogenase,
lactate dehydrogenase, malate dehydrogenase,
glucose-6-phosphate dehydrogenase, glucose oxidase,
and uricase. Other classes of enzymes such as
20   hydrolases, transferases, lyases, and isomerases
can also be employed. It is particularly preferred
that the second label also be an enzyme where a
product of the first enzyme reaction is a substrate
or cofactor for the second enzyme so as to result
25   in rapid enzyme reaction in the environment of the
bound hybrid. For example, where the first enzyme
is an oxidoreductase producing hydrogen peroxide as
a product, the second enzyme can be peroxidase. A
detailed listing and description of useful enzymes
30   is provided in U.S. Patent Nos. 4,233,402 and
4,275,149 incorporated herein by reference.
Other useful systems involve one enzyme as the
first label which catalyses a reaction producing a
prosthetic group for an apoenzyme or proenzyme.

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Nonenzymatic catalysts can also serve as labels as
previously mentioned. By way of exampl , r ference
is made to U.S. Patent No. 4,160,645.

5   Another preferred interaction between the
labels is that of energy transfer. The first label
will be a photoemitting substance such as a
fluorescer or luminescer, the former producing an
emission upon irradiation and the second producing
an emission upon chemical reaction. The
10   photoemission is absorbable by the second label to
either quench the emission or to provide a second
emission such as where the absorbing label is a
fluorescer itself. Pairings of compounds useful
for this effect are described in detail in U.S.
15   Patent Nos. 4,275,149 and 4,318,981. Some
preferred fluorescer/quencher pairs are
naphthalene/anthracene, α-naphthylamine/dansyl,
tryptophan/dansyl, dansyl/fluorescein,
fluorescein/rhodamine, tryptophan/fluorescein,
20   N-[p-(2-benzoxazolyl)phenyl]maleimide
(BPM)/thiochrome,
BPM/8-anilino-1-naphthalenesulfonate (ANS),
thiochrome/N-(4-dimethylamino-3,5-dinitrophenyl)
maleimide (DDPM), and ANS/DDPM. Some preferred
25   luminescer/quencher pairs are luminol with
fluorescein, eosin or rhodamine S.

If desired, various modifications of the
labeled reagents can be employed without departing
from the scope of the present invention. In one
30   variation, one of the labeled reagents comprises a
solid phase to which is linked the binding
substance and separately the label. The solid
phase can be the walls of the reaction container or
a dispersed solid such as a polyacrylamide or

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agarose bead. One could also modify the binding
substance with a bindable ligand such as a hapten
or biotin and introduce the label by addition of a
labeled anti-hapten antibody or labeled avidin

5   before or after the hybridization reaction. Thus,
it can be seen that the label can be linked to the
binding substance directly or indirectly through
intermediary components.

The Binding Reagents

10   A critical aspect of the present invention is
the formation of a hybrid between the probe and the
polynucleotide sequence of interest which comprises
binding sites for the two specific binding
reagents. A principle of the assay is that

15   hybridization of the probe with the desired .
sequence results in a bringing together of the
respective binding sites for the two binding
reagents such that the labels are brought within a
proximate interaction distance of one another. Any

20   design of the system can be used which results in a
binding site for at least one of the two labeled
reagents which is unique to the hybrid. It will
thereby be assured that the desired localization of
the labeling pair will occur only upon formation of

25   the hybrid.
The binding of the labeled reagents to the
hybrid will normally involve a highly specific
noncovalent binding such as is characteristic of a
variety of biologically derived substances,

30   particularly binding proteins such as
immunoglobulins. A variety of binding substances
can be used to provide at least one binding reagent

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which has a unique binding affinity for the hybrid
with inconsequential binding affinity for single
stranded nucleic acids such as unhybridized probe
and unhybridized sample nucleic acids.

5   Particularly preferred binding substances are
antibody reagents having anti-hybrid binding
activity and can be whole antibodies or fragments
thereof, or aggregates or conjugates thereof, of
the conventional polyclonal or monoclonal variety.

10   Preferred antibody reagents will be those that are
selective for binding (i) DNA·RNA or RNA·RNA
hybrids or (ii) intercalation complexes. It is
currently known that antibodies can be stimulated
which are selective for DNA·RNA or RNA·RNA hybrids

15   over the single stranded nucleic acids, however, it
is presently considered infeasible to generate such
selectivity in the case of DNA·DNA hybrids. To the
extent that selective DNA·DNA antibodies are
developed in the future, they will clearly be

20   applicable to the present invention. Antibodies to
DNA·RNA or RNA·RNA hybrids can be used where the
probe is RNA and the sample nucleic acids are DNA
or RNA or where the probe is DNA and the sample
RNA.

25   Further, it should be understood that in
referring to an RNA probe used with an anti-DNA·RNA
or anti-RNA·RNA reagent, it is contemplated herein
that not all nucleotides comprised in the probe be
ribonucleotides, i.e., bearing a 2'-hydroxyl group.

30   The fundamental feature of an RNA probe as used
herein is that it be sufficiently non-DNA in
character to enable the stimulation of antibodies
to DNA·RNA or RNA·RNA hybrids comprising an RNA
probe which do not crossreact to an analytically

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significant degr e with the individual single
strands forming such hybrids. Therefor , one or
more of the 2'-positions on the nucleotides
comprised in the probe can be in the deoxy form
5   provided the antibody binding characteristics
necessary for performance of the present assay are
maintained to a substantial degree. Likewise, in
addition or alternatively to such limited 2'-deoxy
modification, an RNA probe can comprise nucleotides
10   having other 2'-modifications, or in general any
other modification along its ribose phosphate
backbone provided there is not substantial
interference with the specificity of the antibody
to the double stranded hybridization product
15   compared to its individual single strands.

Where such modifications exist in an RNA
probe, the immunogen used to raise the antibody
reagent would preferably comprise one strand having
substantially corresponding modifications and the
20   other strand being substantially unmodified RNA or
DNA, depending on whether sample RNA or DNA is
intended to be detected. Preferably, the modified
strand in the immunogen would be identical to the
modified strand in an RNA probe. An example of an
25   immunogen is the hybrid poly(2'-0-methyladenylic
acid)˙poly(2'-deoxythymidylic acid). Another would
be poly(2'-0-ethylinosinic acid)˙poly(ribocytidylic
acid). The following are further examples of
modified nucleotides which could be comprised in an
30   RNA probe: 2'-0-methylribonucleotide, 2-0
-ethylribonucleotide, 2'-azidodeoxyribonucleotide,
2'-chlorodeoxyribonucleotide, 2'-0
-acetylribonucleotide, and the methyl phosphonates
or phosphorothiolates of ribonucleotides or

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deoxyribonucleotides. Modified nucleotides can
appear in RNA probes as a result of introduction
during enzymic synthesis of the probe from a
template. For example, adenosine

5   5'-0-(1-thiotriphosphate) (ATPαS) and dATPαS are
substrates for DNA dependent RNA polymerases and
DNA polymerases, respectively. Alternatively, the
chemical modification can be introduced after the
probe has been prepared. For example, an RNA probe

10   can be 2'-0-acetylated with acetic anhydride under
mild conditions in an aqueous solvent.

        Immunogens for stimulating antibodies specific
for RNA·DNA hybrids can comprise homopolymeric or
heteropolymeric polynucleotide duplexes. Among the

15   possible homopolymer duplexes, particularly
preferred is poly(rA)·poly(dT) [Kitagawa and
Stollar (1982) Mol. Immunol. 19:413]. However, in
general, heteropolymer duplexes will be preferably
used and can be prepared in a variety of ways,

20   including transcription of ϕX174 virion DNA with
RNA polymerase [Nakazato (1980) Biochem. 19:2835].
The selected RNA·DNA duplexes are adsorbed to a
methylated protein, or otherwise linked to a
conventional immunogenic carrier material, such as

25   bovine serum albumin, and injected into the desired
host animal [see also Stollar (1980) Meth. Enzymol.
70:70]. Antibodies to RNA·RNA duplexes can be
raised against double stranded RNAs from viruses
such as reovirus or Fiji disease virus which

30   infects sugar cane, among others. Also,
homopolymer duplexes such as poly(rI)·poly(rC) or
poly(rA)·poly(rU), among others, can be used for
immunization as above. Further information
regarding antibodies to RNA·DNA and RNA·RNA hybrids

is provid d in commonly assigned U.S. Patent
Application Serial No. 616,132, filed June 1, 1984.

Antibodies to intercalation complexes can be
prepared against an immunogen which will usually
comprise an ionic complex between a cationic
protein or protein derivative (e.g., methylated
bovine serum albumin) and the anionic
intercalator-nucleic acid complex. Ideally, the
intercalator will be covalently coupled to the
double stranded nucleic acid. The
intercalator-nucleic acid conjugate alternatively
can be covalently coupled to a carrier protein.
The nucleic acid portion of the immunogen can
comprise the specific paired sequences found in the
assay hybrid or can comprise any other desirable
sequences since the specificity of the antibody
will generally not be dependent upon the particular
base sequences involved. Further information
regarding antibodies to intercalation complexes is
provided in commonly assigned U.S. Patent
Application Serial No. 560,429, filed December 12,
1983.

As stated above, the antibody reagent can
consist of whole antibodies, antibody fragments,
polyfunctional antibody aggregates, or in general
any substance comprising one or more specific
binding sites from an antibody. When in the form
of whole antibody, it can belong to any of the
classes and subclasses of known immunoglobulins,
e.g., IgG, IgM, and so forth. Any fragment of any
such antibody which retains specific binding
affinity for the hybridized probe can also be
employed, for instance, the fragments of IgG
conventionally known as Fab, F(ab'), and F(ab')$_2$.

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In addition, aggregates, polymers, derivatives and conjugates of immunoglobulins or their fragments can be used where appropriate.

The immunoglobulin source for the antibody
5 reagent can be obtained in any available manner such as conventional antiserum and monoclonal techniques. Antiserum can be obtained by well-established techniques involving immunization of an animal, such as a mouse, rabbit, guinea pig
10 or goat, with an appropriate immunogen. The immunoglobulins can also be obtained by somatic cell hybridization techniques, such resulting in what are commonly referred to as monoclonal antibodies, also involving the use of an
15 appropriate immunogen.

In those instances where an antibody reagent selective for intercalation complexes is employed as one of the binding reagents, a variety of intercalator compounds can be involved. In general
20 it can be said that the intercalator compound preferably is a low molecular weight, planar, usually aromatic but sometimes polycyclic, molecule capable of binding with double stranded nucleic acids, e.g., DNA·DNA, DNA·RNA, or RNA·RNA duplexes,
25 usually by insertion between base pairs. The primary binding mechanism will usually be noncovalent, with covalent binding occurring as a second step where the intercalator has reactive or activatable chemical groups which will form
30 covalent bonds with neighboring chemical groups on one or both of the intercalated duplex strands. The result of intercalation is the spreading of adjacent base pairs to about twice their normal separation distance, leading to an increase in

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molecular length of the duplex. Further, unwinding of the double helix of about 12 to 36 d grees must occur in order to accomodate the intercalator. General reviews and further information can be obtained from Lerman, J..Mol. Biol. 3:18(1961); Bloomfield et al, "Physical Chemistry of Nucleic Acids", Chapter 7, pp. 429-476, Harper and Rowe, NY(1974); Waring, Nature 219:1320 (1968); Hartmann et al, Angew. Chem., Engl. Ed. 7:693(1968); Lippard, Accts. Chem. Res. 11:211(1978); Wilson, Intercalation Chemistry(1982),445; and Berman et al, Ann. Rev. Biophys. Bioeng. 10:87(1981); as well as from the above-referenced U.S. Serial No. 560,429. Exemplary of intercalators are acridine dyes, e.g., acridine orange, the phenanthridines, e.g., ethidium, the phenazines, furocoumarins, phenothiazines, and quinolines.

The intercalation complexes are formed in the assay medium during hybridization by use of a probe which has been modified in its complementary, single stranded region to have the intercalator chemically linked thereto such that upon hybridization the intercalation complexes are formed. Essentially any convenient method can be used to accomplish such linkage. Usually, the linkage is formed by effecting intercalation with a reactive, preferably photoreactive intercalator, followed by the linking reaction. A particularly useful method involves the azidointercalators. Upon exposure to long wavelength ultraviolet or visible light, the reactive nitrenes are readily generated. The nitrenes of arylazides prefer insertion reactions over their rearrangement products [see White et al, Methods in Enzymol.

46:644(1977)]. Representative azidointercalators are 3-azidoacridine, 9-azidoacridine, ethidium monoazides, ethidium diazide, ethidium dimer azide [Mitchell et al, JACS 104:4265(1982)],

5 4-azido-7-chloroquinoline, and 2-azidofluorene. Other useful photoreactable intercalators are the furocoumarins which form [2+2] cycloadducts with pyrimidine residues. Alkylating agents can also be used such as bis-chloroethylamines and epoxides or

10 aziridines, e.g., aflatoxins, polycyclic hydrocarbon epoxides, mitomycin, and norphillin A. The intercalator-modified duplex is then denatured to yield the modified single stranded probe.

Repeating what is stated previously, at least

15 one of the binding reagents must be of the type discussed above, however, there is a greater latitude of choice possible for the other binding reagent since it need not discriminate between the hybrid and other nucleic acids, including the probe

20 and sample materials, to accomplish the desired effect. Accordingly, the second binding reagent can be the same or a different binding material as used in the aforesaid reagent except that when the same, it is, of course, ultimately different as an

25 assay reagent by being labeled with the second label. When employing an antibody reagent selective for binding intercalation complexes for both binding reagents, it will normally be desired to employ a probe which has been modified to have the

30 intercalator chemically linked to the probe, as opposed to being added as a soluble compound.

Alternatively, the second binding reagent can be any material which binds the probe. Thus, it can be an antibody reagent that binds to nucleic

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acids nonspecifically such as anti-DNA·DNA.
Anoth r mbodiment will employ a probe comprising a
specific binding ligand moiety with th nonspecific
binding reagent then being a binding partner for
such ligand moiety. In such case, the ligand
moiety/binding partner pair can be selected from
such known materials as haptens/antibodies,
antigens/antibodies, biotin/avidin,
lectins/polysaccharides, hormones/receptor
proteins, and the like. In this respect, ·reference
is made to British Pat. 2,019,408; European Patent
Applns. 63,879 and 97,373 and PCT Published Applns.
83-1459 and 83-2286.

     While it is generally preferred that the
critical microenvironment of the bound hybrid be
created in a portion or the entirety of the
hybridized complementary region of the sample/probe
duplex, in principle such microenvironment can also
occur at the boundary of such complementary region
and the flanking region. This can occur employing
binding reagents as discussed above. Additionally,
one can use as the nonspecific binding reagent a
binding substance for a binding site on double
stranded flanking regions of the probe. Examples
of such binding site/binding partner pairs are
promotor sequences (e.g., lac-promotor,
trp-promoters, and promoters associated with
bacteriophages)/polymerase; lac operator/lac
repressor protein; and antigenic nucleotides and
sequences (e.g., Z-DNA and rare nucleotides such as
5-halodeoxyuridines)/antibodies thereto.

     With reference to the drawings and the
examples which follow, a few specific embodiments
of the present assay scheme can be described.

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The method depicted in Fig. 1 employs an
unmodified polynucleotide probe which is either RNA
or DNA when the sample sequence of interest is RNA
or when the sample sequence is DNA the probe is
RNA. The binding reagents are the same or
different antibody reagents (Ab) selective for
RNA·DNA or RNA·RNA hybrids as the case may be. One
antibody reagent is labeled with glucose oxidase
(GOD) and the other with peroxidase (POD). In the
presence of glucose and a color indicator for
hydrogen peroxide, the rate of color development
will be dependent upon the extent of hybridization
that takes place. In the bound hybrid, glucose
oxidase and peroxidase are brought within a
distance a which results in an increased rate of
color development due to rapid diffusion of
hydrogen peroxide to peroxidase. In the bulk
medium, hydrogen peroxide must on average diffuse
distance b in order to cause a color development
event which reflects in a significantly different
rate. Optionally, catalase can be included in the
bulk solution at concentrations sufficient to
effectively destroy all hydrogen peroxide that
diffuses away from the microenvironment of the
bound hybrid to reduce background color formation.

The use of a ligand-modified probe is
illustrated in Fig. 2, with biotin (Bio) being the
selected representative ligand. In this case the
GOD-labeled binding reagent is again an antibody to
RNA·DNA or RNA·RNA as the case may be, and the
other binding reagent is POD-labeled avidin (Av).
The color development readout will be understood as
in the case of method A.

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In the method shown in Fig. 3, the probe is
doubly modifi d, with both biotin as in method B
and a chemically linked intercalator (I). An
antibody to intercalation complexes involving I is
5   labeled with a fluorescer (F) and avidin is labeled
with a quencher (Q). The fluorescer and quencher
labels are brought into energy transfer distance a
in the bound hybrid such that upon irradiation with
light of a first wavelength ($h\nu_1$), the emitted
10   energy ($h\nu_2$) is absorbed by the quencher and not
detected. On the other hand, fluorescer-labeled
antibody in the bulk solution remains on average
distance b from the quencher, which is not close
enough for efficient energy transfer, and the
15   fluorescence emission is observed. The amount of
$h\nu_2$ light detected is inversely related to the
amount of hybridization that occurs.

Reaction Mixture

The test sample to be assayed can be any
medium of interest, and will usually be a liquid
sample of medical, veterinary, environmental,
5   nutritional, or industrial significance. Human and
animal specimens and body fluids particularly can
be assayed by the present method, including urine,
blood (serum or plasma), amniotic fluid, milk,
cerebrospinal fluid, sputum, fecal matter, lung
10   aspirates, throat swabs, genital swabs and
exudates, rectal swabs, and nasopharnygal
aspirates. Where the test sample obtained from the
patient or other source to be tested contains
principally double stranded nucleic acids, such as
15   contained in cells, the sample will be treated to
denature the nucleic acids, and if necessary first
to release nucleic acids from cells. Denaturation
of nucleic acids is preferably accomplished by
heating in boiling water or alkali treatment (e.g.,
20   0.1 N sodium hydroxide), which if desired, can
simultaneously be used to lyse cells. Also,
release of nucleic acids can, for example, be
obtained by mechanical disruption (freeze/thaw,
abrasion, sonication), physical/chemical disruption
25   (detergents such as Triton, Tween, sodium
dodecylsulfate, alkali treatment, osmotic shock, or
heat), or enzymatic lysis (lysozyme, proteinase K,
pepsin). The resulting test medium will contain
nucleic acids in single stranded form which can
30   then be assayed according to the present
hybridization method. In those situations where
RNA·DNA hybrids are to be detected with labeled

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antibody reagents, mRNA and rRNA in the sample can
be removed from participating in the binding
reactions by conventional methods such as treatment
with alkaline conditions, e.g., the same conditions

5   used to denature the nucleic acids in the sample.

As is known in the art, various hybridization
conditions can be employed in the assay.
Typically, hybridization will proceed at slightly
elevated temperatures, e.g., between about 35 and

10   75°C and usually around 65°C, in a solution
comprising buffer at pH between about 6 and 8 and
with appropriate ionic strength (e.g., 5XSSC where
1XSSC = 0.15M sodium chloride and 0.015M sodium
citrate, pH 7.0). In cases where lower

15   hybridization temperatures are desirable, hydrogen
bonding reagents such as dimethyl sulfoxide and
formamide can be included. The degree of
complementarity between the sample and probe
strands required for hybridization to occur depends

20   on the stringency of the conditions. Factors which
determine stringency are known in the art.

Normally, the temperature conditions selected
for hybridization will be incompatible with the
binding of the anti-hybrid reagent to formed

25   hybrids and detection of the label response.
Accordingly, the anti-hybrid binding step and label
detection step will proceed after completion of the
hybridization step. The reaction mixture will
usually be brought to a temperature in the range of

30   from about 3°C to about 40°C and the binding and
detection steps then performed. Dilution of the
hybridization mixture prior to addition of the
antibody reagent is desirable when the salt and/or
formamide concentrations are high enough to

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interfere significantly with the antibody binding
reaction.

Reagent System

The present invention additionally provides a
reagent system, i.e., reagent combination or means,
comprising all of the essential elements required
to conduct a desired assay method. The reagent
system is presented in a commercially packaged
form, as a composition or admixture where the
compatability of the reagents will allow, in a test
device configuration, or more usually as a test
kit, i.e., a packaged combination of one or more
containers, devices, or the like holding the
necessary reagents, and usually including written
instructions for the performance of assays.
Reagent systems of the present invention include
all configurations and compositions for performing
the various hybridization formats described herein.

In all cases, the reagent system will comprise
(1) a nucleic acid probe as described herein, and
(2) the first and second labeled binding reagents.
A test kit form of the system can additionally
include ancillary chemicals such as the components
of the hybridization solution and denaturation
agents capable of converting double stranded
nucleic acids in a test sample into single stranded
form. Preferably, there is included a chemical
lysing and denaturing agent, e.g., alkali, for
treating the sample to release single stranded
nucleic acid therefrom.

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The present invention will now be illustrated, but is not intended to be limited, by the following examples.

Example I

5   Hybridization Assay for Bacterial RNA
         ·Monitored with Labeled Antibodies
                 to RNA·DNA Hybrid

         A. Monoclonal antibody to RNA·DNA hybrid is
     raised by the method of Stuart et al (1981) PNAS
10   78, 3751. Antibody is isolated from ascites fluid
     by affinity chromatography on protein A Sepharose
     available from Pharmacia Fine Chemicals,
     Piscataway, NJ. About 1 milliliter (ml) of ascites
     fluid per ml of protein A Sepharose is applied to
15   the column and then the column is washed with 50
     millimolar (mM) Bicine buffer, pH 8.0, 0.15 molar
     (M) NaCl until the absorbance of the effluent at
     280 nanometers (nm) is less than 0.05. The bound
     antibody is eluted with 0.1M glycine buffer, pH 3.0
20   and antibody is detected by measurement of
     absorbance at 280 nm. The antibody pool is
     adjusted to pH 5.0 or above by addition of 1M
     Bicine buffer, pH 8.5. The pool is dialyzed into
     0.1M borate buffer, pH 9.0
25   The antibody is labeled lightly with
     5-(4,6-dichlorotriazin-2-yl)-aminofluorescein
     (DTAF) (Molecular Probes, Inc, Junction City,
     Oregon) by the method of Blakeslee and Baines
     (1976) J. Immunol. Meth. 13, 305. A solution
30   containing 1.44 micromoles (µmoles) of DTAF in 361
     microliters (µL) of borate buffer is added to 2.4

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ml f 0.014 mM antibody and allow d to react for one hour at 25°C. The reaction mixture is chromatographed on a 1 x 25 cm Biogel P-6DG (BioRad Laboratories, Richmond, CA) column with 0.1M sodium phosphate buffer, pH 7.0, 0.1M NaCl as the eluent. Absorbances at 280 nm of 1 ml fractions are monitored and those comprising the first peak are pooled. The molar DTAF/protein ratio is determined by the method of The and Feltkamp (1970) Immunology 18, 865.

Free thiol groups are introduced into the antibody by reaction with N-succinimidyl 3-(2-pyridyldithio)propionate and subsequent mild reduction with dithiothreitol [Carlson et al (1978) Biochem. J. 173, 723].

B.   A glucose oxidase antibody conjugate is prepared as follows. Maleimide groups are introduced onto glucose oxidase (available from Miles Scientific, Naperville, IL) by reaction with the N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide (SMCC) as described by [Yoshitoake, et al, (1979) Eur. J. Biochem. 101, 395 (1979)]. This glucose oxidase is immediately combined with a two-fold molar excess of the thiol derivatized antibody described above. The reaction conditions and purification methods of Yoshitake et al are followed. Enzyme content of the purified conjugate is determined by measurement of enzyme activity and the antibody content is determined by measurement of the DTAF content as described by The and Feltkamp, *supra*.

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C.   Horse radish peroxidase is reacted with
SMCC and coupled to the thiol derivatized antibody
by the method of Ishikawa et al (1983) J.
Immunoassay 4:209.

5   D.   A 565 base pair fragment from a gene for
the 16s ribosomal RNA from E. coli has been cloned
between Hind III sites of a pBR322 vector [Brosius
(1978) Proc. Nat'l. Acad. Sci. 75:4801]. The
fragment is excised by digestion with Hind III
10   restriction endonuclease.

E.   A urine specimen containing greater than
10^5 bacteria per ml is diluted three-fold into an
enriched culture broth and incubated at 35°C until
the cell population reached about 10^9 organisms per
15   ml. Aliquots of the culture are transferred to a
series of tubes to give 10^5 to 10^9 bacteria per
tube. The tubes are centrifiged at 10,000 x g for
10 minutes and the supernatants are discarded. The
cells are lysed in 20 µl per tube of 10 milligrams
20   (mg) egg white lysozyme (Sigma Chemical Co., St.
Louis, MO) in 10 mM Tris-hydrochloride buffer, pH
8.0, 0.1M NaCl and 5 mM ethylenediaminetetraacetic
acid. Fifteen minutes later, 80 µl of a solution
composed of 60% formamide and 40% 0.16 M sodium
25   phosphate buffer, pH 6.5, 1.44 M NaCl, and 0.1%
(w/v) sodium dodecylsulfate.
The DNA probe described in section D above, in
0.2M sodium phosphate buffer, pH 6.5, is denatured
in a boiling water bath for 5 minutes and cooled on
30   ice. Twenty microliter aliquots (80 ng DNA) are
added to each tube and incubated at 55°C for 18
hours. Then 500 µl of peroxidase labeled antibody

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and glucose oxidas labeled antibody are add d to each tube and allowed to stand at room temperature for 2 hours. The concentrations of these enzyme conjugates are optimized in preliminary experiments

5   to give optimum signal to background ratios.

The following substrate reagent is prepared and used within 3 hours: 0.1M sodium phosphate, pH 6.5, 0.1% bovine serum albumin, 20 mM 3,5-dichloro-2-hydroxybenzene sulfonate, 0.1M

10   glucose, 0.01 unit catalase/ml and 0.2 mM 4-aminoantipyrine. One milliliter of substrate reagent is added to each tube and incubated at 37°C for 30 minutes. The the absorbances at 510 nm are recorded. As the number of bacteria per tube

15   increases the amount of ribosomal RNA·DNA hybrid will increase and as a result the absorbance will be increased.

Example II

Hybridization Assay for Detection of Bacterial

Ribosomal RNA Using a Biotinylated
DNA Probe

5   A.   Biotin-ll-dUTP and streptavidin-horse
radish peroxidase conjugate are available from Enzo
Biochem., Inc., New York, NY.

           B.   The 565 base pair probe for ribosomal RNA
is labeled with biotin-ll-dUTP by the method of
10   Langer et al (1981) Proc. Nat'l. Acad. Sci.,
78:6633.

           C.   Bacteria are processed as outlined in
Example I, section E above. The lysates are
combined with 80 µl of a solution composed of 60%
15   formamide and 40% 0.16 M sodium phosphate buffer,
pH 6.5, 1.44 M NaCl and 0.1% (w/v) sodium
dodecylsulfate. The biotinylated DNA probe is
denatured in a boiling water bath for 5 minutes and
then cooled on ice. The probe is in 0.2M sodium
20   phosphate buffer, pH 6.5 and 20 $^{\mu}$l aliquots
containing 80 ng probe are added to each extract
and incubated at 65°C for 18 hours. Then 500 $^{\mu}$l of
peroxidase labeled streptavidin and glucose oxidase
labeled antibody against RNA·DNA hybrid is added to
25   each tube and allowed to stand at room temperature
for 2 hours. The concentrations of these enzyme
conjugates are optimized in preliminary experiments
to give maximum signal to background ratios.

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Th   nzyme reactions ar initiated by addition
of 1 ml f the substrate r agent d scribed in
Example I, section D. Thes reacti ns are allowed
to proceed at 37°C for 30 minutes and then the
absorbances at 510 nm are recorded. The
absorbances will increase as the number of bacteria
added per tube increases.

Example III

Hybridization Assay for Bacterial Ribosomal
RNA Using a Probe Intercalated with Ethidium
Bromide

A.   Calf thymus DNA (Sigma Chemical Co.) is
treated with pronase to digest protein contaminants
and then precipitated in ethanol. The DNA is
dissolved in 20 mM tris-hydrochloride buffer, pH
8.0, 0.2M NaCl.

A photoreactive derivative of ethidium
bromide, 8-azidoethidium, is prepared by the method
of Graves et al (1977) Biochim. Biophys. Acta,
479:98. A solution is prepared containing about 1
mM base pairs of the DNA and 0.5 mM
8-azidoethidium. This solution is stirred in a
glass reaction vessel immersed in a water bath
maintained at 20-30°C and is photolyzed 10 to 20 cm
from a 150 watt spotlight for 60 minutes.
Following the photolysis the noncovalently bound
ethidium derivatives are removed by extractions
with water saturated *n*-butanol. The amount of
ethidium coupled to the DNA is estimated using the
extinction coefficients of $E_{490} = 4 \times 10^3 M^{-1} cm^{-1}$
for photolyzed ethidium azide, the relationship

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between A_{260} and A_{490} for photolyzed ethidium bound to DNA $[A_{260} = (A_{490} \times 3.4)-0.011]$, and $E_{260} = 1.32 \times 10^4 \ M^{-1} cm^{-1}$ for the concentration of DNA base pairs of a given DNA being labeled.

5   B.   Preparation of methylated thyroglobulin
      One hundred milligrams of bovine thyroglobulin (Sigma) is combined with 10 ml of anhydrous methanol and 400 µl of 2.55 M HCl in methanol. This mixture is stirred on a rotary mixer at room
10   temperature for 5 days. The precipitate is collected by centrifugation and washed twice with methanol and twice with ethanol. Then it is dried under vacuum overnight. About 82 mg of dry powder is obtained.

15   C.   An immunogen is prepared by combining the DNA-ethidium complex with methylated thyroglobulin as outlined by Stbller (1980) Methods in Enzymology 70:70. Twenty to fifty micrograms of the immunogen in Freunds adjuvant are injected per mouse and
20   booster injections are given weekly beginning two weeks after the original immunization. Antibody titers are measured by an ELISA procedure and hybridomas are produced and screened for antibody by standard procedures [Galfre and Milstein, (1981)
25   Meth. Enzymol., 73:1; Poirier, et al (1982) Proc. Nat'l. Acad. Sci., 79:6443]. Selection procedures were designed for antibodies which bind the DNA-ethidium complex with association constants of 10^{10} or greater but do not bind to single or double
30   stranded DNA or the ethidium residue alone.
      Antibody is isolated from ascites fluid as outlined in Example I, section A for antibody to

RNA·DNA hybrid. This antibody is also label d
lightly with DTAF and coupled to glucos oxidase.

D.   The 565 base pair fragment from the
pKK115 plasmid is cloned into the Hind III site of
M13 mp9 [Messing and Vieria, (1982) Gene 19, 269;
commercially available from New England Biolabs,
Beverly, MA]. The fragment inserts in two
orientations and a clone with the virion DNA
sequence complementary to ribosomal RNA is selected
for further work.
     The modified M13 mp9 is propagated in E. coli
JM103 [Δlac, pro)thi, strA, supE, endA, sbcB15,
hsdR4, F'traD36, proAB, lacIq, ZΔM15] available
from Bethesda Research Laboratories, Gaithersburg,
MD. The phage is isolated from culture broth by
precipitation with polyethylene glycol and the
virion DNA is purified by phenol/chloroform
extraction [Maniatis et al (1982) Molecular
Cloning, A Laboratory Manual, Cold Spring Harbor].
     An oligonucleotide primer (CACAATTCCACACAAC;
available from New England Biolabs) is
complementary to the M13 mp9 vector on the 5'-OH
side of the inserted ribosomal RNA probe. This
primer is used to initiate replication of the M 13
sequence by E. coli DNA polymerase (Klenow
fragment) in the presence of limiting quantities of
deoxynucleotides triphosphates. The replication is
limited to an extent that the inserted ribosomal
RNA probe remains single stranded and available for
hybridization [Hu and Messing (1982) Gene 17:271].
     This modified M 13 probe is mixed with
8-azidoethidium, photolyzed and purified as
outlined in section A above.

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E.   Bacteria are prepared and lysed as
described in Example I, part A. The lysates are
mixed with 80 µl of the formamide hybridization
solution described in Example I, Part E. Twenty
5   microliters of the ethidium-probe complex (100 ng
DNA) (section D) is added to the extracts and
incubated at 65°C for 18 hours and then 500 µl of
peroxidase labeled antibody to RNA·DNA and glucose
oxidase labeled antibody to ethidium-DNA complex is
10   added to each tube. The mixtures are allowed to
stand at room temperature for 2 hours.

At the end of this incubation, 1.0 ml of the
substrate reagent, Example I, part E, is added and
incubated at 37°C for 30 minutes. Then the
15   absorbance at 510 nm is recorded. As the number of
bacteria per tube is increased, the absorbance will
increase.

Example IV

Hybridization Assays for Cylomegalovirus
20   Monitored with a Fluorescer-Quencher Pair

A.   A 1500 base pair fragment of the
cytomegalovirus genome is cloned into the M13mp8
vector. A complementary copy of the inserted
fragment is synthesized *in vitro* with DNA
25   polymerase in the presence of a biotinylated
nucleotide triphosphate to provide a biotinylated
probe. This probe is modified further by covalent
incorporation of ethidium residues as intercalation
complexes.

30   An EcoRI digest of cytomegalovirus DNA is
prepared and the fragments are cloned into the

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plasmid pACYC184 [Tamashiro et al (1982) J.
Virology 42:547]. The plasmids are propogated in
E. coli strain HB101 Rec A⁻ and the EcoRI e
fragment described in the Tamashiro reference is
used for preparation of the probe. The fragment is
removed from the plasmid by digestion with EcoRI
restriction enzyme and cloned into the EcoRI site
of the M13mp8 vector (New England Biolabs) which is
propagated in the E. coli K12JM101 host. The virus
is isolated from the culture fluid by precipitation
with polyethyleneglycol and the DNA is purified by
phenol/chloroform extraction.

The DNA is annealed with a 17 base primer with
the sequence GTAAAACGACGGCCAGT (New England
Biolabs) which is complementary to a region of the
M13mp8 sequence near the 3'-OH end of the EcoRI e
insert. The DNA is 20 mM Tris-hydrochloride
buffer, pH 8.0, containing 10 mM $MgCl_2$ is combined
with a molar excess of the primer and incubated at
55°C for 45 minutes [Bankier and Barrell (1983)
Techniques in Nucleic Acid Biochemistry, Elsevier,
Ireland]. Then the reaction mixture is made 15 mM
in dATP, dCTP, dGTP and Bio-11-dUTP (available from
Enzo Biochem) and finally the Klenow fragment of
DNA polymerase I is added. This reaction is
incubated at 25°C for a period determined in
preliminary experiments. In these experiments
samples are taken from the reaction mixture at
various times and electrophoresed in alkaline
agarose gel [Maniatis et al, supra]. The objective
is to find reaction conditions which give biotin
labeled probes that extend through the EcoRI e
insert. This procedure will provide biotin labeled
DNA with some variation in length; however,

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extension of the labeled DNA beyond the EcoRI e
insert into the M13 sequence is acceptable for the
present purpose.

The next step is the covalent coupling of
ethidium residues to the biotin labeled DNA as
intercalation complexes. This is accomplished by
photolysis of the DNA with 8-azidoethidium prepared
by the method of Graves et al, supra. The biotin
labeled DNA is extracted with phenol/chloroform and
precipitated with ethanol. It is dissolved in 20
mM Tris-hydrochloride buffer, pH 8.0, 0.2 M NaCl,
to give about 50 μg DNA/ml and made 0.5 mM with
8-azidoethidium. The mixture is photolyzed for 1
hour 10 to 20 cm from a 150 watt spotlight. The
mixture is stirred during this period in a glass
reaction vessel immersed in a glass water bath.
The bath maintains a reaction temperature of 20 to
30°C.

Then the noncovalently bound 8-azidoethidium
and photolysis by-products are removed by the
successive extractions with water saturated
n-butanol. The DNA is precipitated with ethanol
and dissolved in the Tris-buffer. The covalently
bound ethidium is measured spectrophotometrically
as described in Example III, section A.

8-Azidoethidium binds covalently to the DNA
almost exclusively in the double stranded region
where intercalation complexes can form. The
objective is to covalently couple one ethidium
residue per 10 to 50 base pairs. Since the
photolytic reaction goes nearly to completion in
one hour, the incorporation can be decreased by
reducing the reaction time or by reducing the
8-azidoethidium concentration. The incorporation

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can be increased by repeating the photolysis with
fresh 8-azidoethidium.

5   The final step is separation of the biotin
labeled/ethidium modified probe from the M13mp8
template. The labeled probe is substantially
smaller than the M13mp8 template and can be
isolated by alkaline agarose gel electrophoresis
[Maniatis et al, supra]. The gel containing the
10   probe is cut from the agarose gel slab and
recovered by electroelution as described in the
Maniatis reference.

     B.   Fluorescein labeled antibody to
ethidium-DNA intercalation complex.

15   Monoclonal antibody to ethidium-DNA complex
described in Example III, part B above is dialyzed
into 0.1 M sodium borate buffer, pH 9.0, and 0.5 ml
containing 5 mg antibody is combined with 0.5 ml of
10 mM 5(4,6-dichlorotriazin-2-yl)-aminofluorescein
20   (Molecular Probes, Inc., Junction City, OR) in the
borate buffer. The mixture is allowed to stand for
7 hours at room temperature and then it is
chromotographed on a 1 x 26 cm column of Biogel
P6DG resin (Bio-Rad Laboratories) in 0.1 M
25   Tris-hydrochloride buffer, pH 8.0. The absorbances
at 280 nm of 1.0 mL fractions are measured and
fractions from the first eluted peak are pooled.
The fluorescein/protein ratio is measured
spectrophotometrically by the method of The and
30   Feltkamp (1970) Immunology 18:865.

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C.   Preparation of streptavidin labeled with
       4,5-dimethoxy-6-carboxyfluorescein.

The N-hydroxysuccinimide ester of
4',5'-dimethoxy-6-carboxyfluorescein is synthesized
5   by the method of Khanna and Ullman (1980) Anal.
Biochem. 108:156.   (The synthesis actually gives a
mixture of the 5 and 6- carboxyfluorescein
isomers).   Streptavidin isolated from Streptomyces
avidini [Hoffman et al (1980) Proc. Nat'l. Acad.
10   Sci. 77:4666] is labeled with the
N-hydroxysuccinimide ester of
4',5'-dimethoxy-6-carboxyfluorescein by the method
described by Khanna and Ullman for labeling
immunoglobulins.

15   D.   Hybridization assay for cytomegalovirus
             in urine.

The procedure uses centrifugation to isolate
the virus from urine and the viral DNA is
hybridized in solution with the biotin
20   labeled/ethidium modified probe.   Then the labeled
avidin and antibody to ethidium-DNA intercalation
complex are allowed to bind to the hybrids formed
and fluorescence is used to estimate the amount of
hybrid present.
25   Cellular and particulate materials are removed
from urine by centrifugation in a Sorvall GLC-3
centrifuge at 3000 rpm for five minutes.   The
supernatant is placed in a polyallomer
ultracentrifuge tube and run at 25,000 rpm in a
30   Beckman Ti50 rotor for 75 minutes.   The pellets are

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dissolv d in 0.1 M NaOH and incubated at 37°C for 30 minut s.

One hundred fifty microliters of 0.2 M sodium phosphate buffer, pH 6.0, containing 1.8 M NaCl, 0.1% sodium dodecylsulfate (w/v) and 1 mM EDTA is added. Then 20 µL of the biotin labeled/ethidium modified probe (50 mg) is added and the hybridization mixture is incubated at 65°C for 10 hours. After the hybridization reaction, 650 µL of 0.1 M Tris-hydrochloride buffer, pH 8.2, containing the labeled avidin and antibody to ethidium–DNA intercalation complex is added. The concentrations of the labeled proteins in this reagent are optimized in preliminary experiments to maximize the fluorescence quenching to background ratio. The protein binding reactions are allowed to proceed at room temperature for 1 hour.

Then the fluorescence of the reaction mixture is recorded using 495 nm light for excitation and 519 nm for emission. A reference assay is run in parallel with a urine known to be free of cytomegalovirus and the fluorescence signal obtained with this reference assay will be higher than that for samples containing the virus, thus the fluorescence signal will increase as the virus level decreases.

The present invention has been particularly described and exemplified above. Obviously many other variations and modifications of the invention may be made without departing from the spirit and scope thereof.

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PROPOSED CLAIMS FOR EPO, DENMARK,
FINLAND, IRELAND, AND NORWAY

1.   A method for detecting a particular polynucleotide sequence in a test sample comprising single stranded nucleic acids, comprising the steps of contacting the test sample with a nucleic acid probe comprising at least one single stranded base sequence that is substantially complementary to the sequence to be detected, and determining the resulting hybrids,

characterized in that the hybrids are formed to have binding sites for two specific binding reagents, any hybrid that is formed is contacted with the two specific binding reagents, one of which comprises a first label and the other comprising a second label, interaction between the first and second labels providing a detectable response which is measurably different when the two labeled reagents are both bound to the same hybrid, compared to when the two labeled reagents are not so bound, and the detectable response is measured as a function of the presence of the sequence to be detected in the sample.

2.   The method of Claim 1 wherein the first label participates in a first chemical reaction that produces a diffusible product which is a participant in a second chemical reaction with the second label to produce a product that provides the detectable response, the first and second labels preferably being catalysts for the first and second chemical reactions, respectively.

3.   The method of Claim 1 wherein the first and second labels participate in an energy transfer interaction.

4.   The method of Claim 1 wherein one of the specific binding reagents is an antibody reagent.

5. Th method of Claim 4 wh rein the antibody reagent
is one that is selective for binding DNA·RNA hybrids wherein
one of the probe and the sequence to be detected is DNA and
the other is RNA, or is one that is selective for binding
RNA·RNA hybrids wherein both the probe and the sequence to
be detected are RNA.

6. The method of Claim 4 wherein the antibody reagent
is one that is selective for binding intercalation complexes
wherein the duplexes formed in the assay comprise a nucleic
acid intercalator bound thereto in the form of intercalation
complexes, preferably the intercalator being chemically
linked to the probe in its single stranded complementary
region whereby upon hybridization with the sequence to be
detected said intercalation complexes are formed in the
resulting hybrid.

7. The method of either of Claim 5 or 6 wherein (i)
the other specific binding reagent comprises the same
antibody reagent, (ii) the nucleic acid probe comprises a
specific binding ligand moiety and the other specific
binding reagent is a binding partner for such ligand moiety,
or (iii) the nucleic acid probe comprises a double stranded
portion having a binding site for a protein and the other
specific binding reagent is such protein.

8. The method of Claim 1 wherein the test sample
comprises a biological sample which has been subject to
conditions to release and denature nucleic acids present
therein.

9. A reagent system for detecting a particular
polynucleotide sequence in a test sample, comprising a
nucleic acid probe comprising at least one single stranded
base sequence that is substantially complementary to the
sequences to be detected,

characterized by comprising additionally first and
second specific binding reagents capable of binding to
hybrids formed between the sequence to be detected in the
sample and the probe, such binding reagents comprising first
and second labels, respectively, which interact with each
other to provide a detectable response which is measurably
different when the two labeled reagents are both bound to
the same hybrid compared to when the two labeled reagents
are not so bound.

10. The reagent system of Claim 9 wherein the first
label participates in a first chemical reaction that
produces a diffusible product which is a participant in a
second chemical reaction with the second label to produce a
product that provides the detectable response, the first and
second labels preferably being catalysts for the first and
second chemical reactions, respectively.

11. The reagent system of Claim 9 wherein the first
and second labels participate in an energy transfer
interaction.

12. The reagent system of Claim 9 wherein one of the
specific binding reagents is an antibody reagent.

13. The reagent system of Claim 12 wherein the
antibody reagent is one that is selective for binding
DNA·RNA hybrids wherein one of the probe and the sequence to
be detected is DNA and the other is RNA, or is one that is
selective for binding RNA·RNA hybrids wherein both the probe
and the sequence to be detected are RNA.

14. The reagent system of Claim 12 wherein the
antibody reagent is one that is selective for binding
intercalation complexes wherein the duplexes formed in the
assay comprise a nucleic acid intercalator bound thereto in

MS-1353

the form of intercalation complexes, preferably the
intercalator being chemically linked to the probe in its
single stranded complementary region whereby upon
hybridization with the sequence to be detected said
intercalation complexes are formed in the resulting hybrid.

15. The reagent system of either of Claims 13 or 14
wherein (i) the other specific binding reagent comprises the
same antibody reagent, (ii) the nucleic acid probe comprises
a specific binding ligand moiety and the other specific
binding reagent is a binding partner for such ligand moiety,
or (iii) the nucleic acid probe comprises a double stranded
portion having a binding site for a protein and the other
specific binding reagent is such protein.

/mc

COMBINE:

1. SINGLE STRANDED SAMPLE NUCLEIC ACIDS

(S)

2. POLYNUCLEOTIDE PROBE
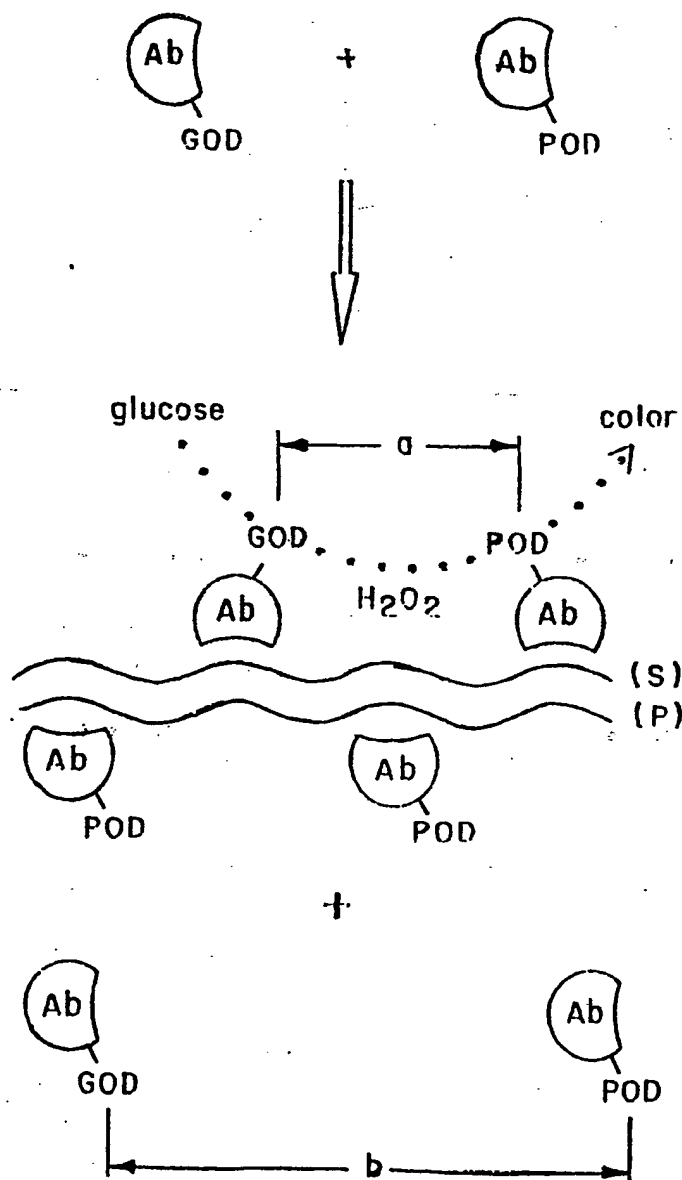
(P)

3. ENZYME LABELED ANTIBODIES

Ab
GOD
+
Ab
POD

glucose $\qquad$ a $\qquad$ color

GOD · · · · · · · POD
H_2O_2

Ab   Ab

(S)
(P)

Ab   Ab
POD   POD

+

Ab
GOD
Ab
POD

b

FIG. 1

COMBINE:

1. SINGLE STRANDED SAMPLE NUCLEIC ACIDS

~~~~~~~~~~~~~~ (S)

2. BIOTIN - MODIFIED POLYNUCLEOTIDE PROBE

~~~~~~~~~~~~~~ (P)
   Bio   Bio   Bio

3. ENZYME - LABELED ANTIBODY AND AVIDIN
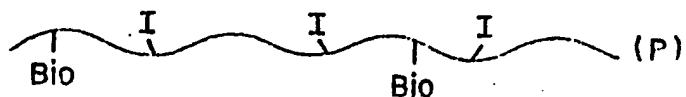

FIG. 2

COMBINE:

I. SINGLE STRANDED SAMPLE NUCLEIC ACIDS

(S)

2. INTERCALATOR / BIOTIN – MODIFIED PROBE

(P)
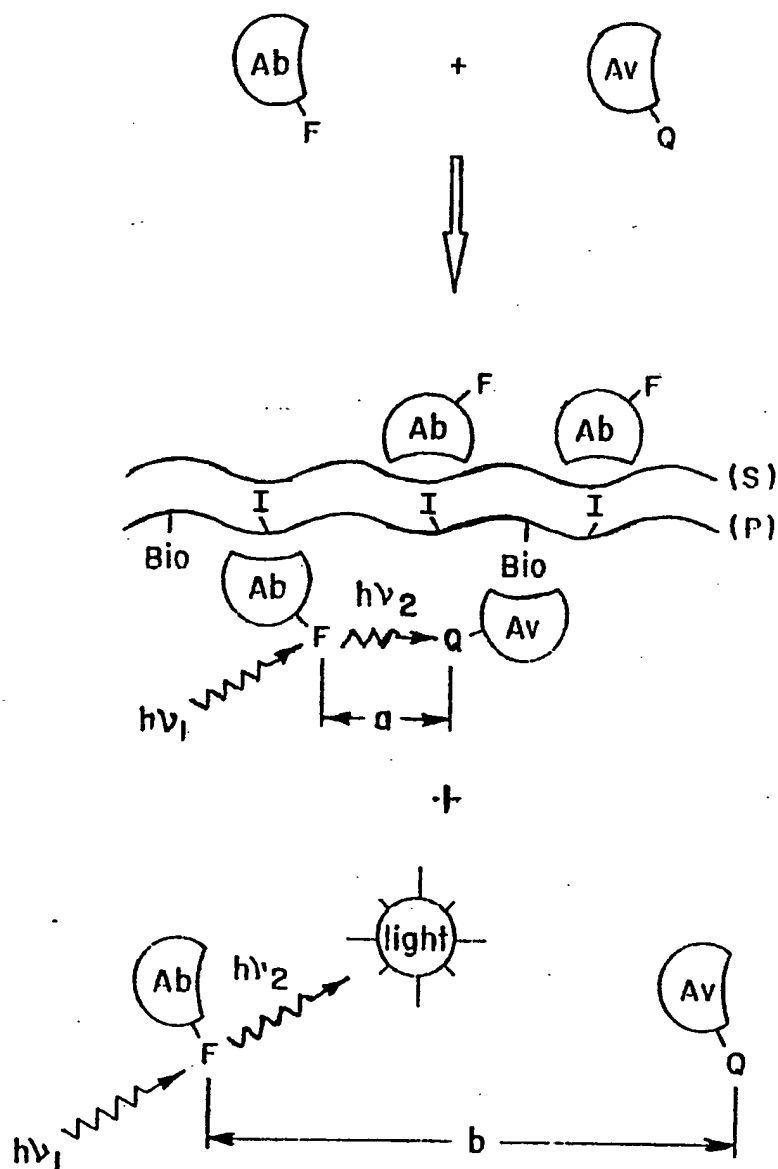
3. ENERGY TRANSFER – LABELED ANTIBODY AND AVIDIN

FIG. 3